REMARKS

Applicants appreciate the call from the Examiner on August 27, 2004 that claims reciting cell lines would be allowable. Without acquiescing in any rejection, applicants have amended the claims without prejudice in order to advance the application towards allowance after final rejection. Applicants reserve the right to file a continuation application(s) with claims that recite, *inter alia*, classes of cell lines, different source types and different combinations of cell lines.

Cell line PNT-2 has been deposited at the European Collection of Cell Cultures, and has been assigned ECACC Ref. No. 95012613. See the captioned application at page 4, last full paragraph. Cell line LNCap is available from the American Type Culture Collection, and has been assigned ATCC number CRL-1740. See the enclosed printout from the ATCC web site. Cell line NIH-1542 is available from the U.S. Government through the National Cancer Institute. See Nishimua *et al., Cancer Res.* 59: 6230-38 (1999) (enclosed); U. S. Patent No. 6,699,483 at Table 1 (enclosed); Bright *et al., Cancer Res.* 57: 995-1002 (1997) (enclosed).

REQUESTED RELIEF

Applicants respectfully request entry of the amendments, which were made without prejudice, and allowance of the claims. The examiner is invited to contact the undersigned should there be any questions.

Respectfully submitted,

September 10, 2004
Date

John P. Isacson
Attorney for Applicants
Registration No. 33,715

HELLER EHRMAN WHITE &
MCAULIFFE
1666 K Street, N.W., Suite 300
Washington, DC 20006
Telephone: (202) 912-2000
Facsimile: (202) 912-2020
Customer No. 26633

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Generation and Genetic Characterization of Immortal Human Prostate Epithelial Cell Lines Derived from Primary Cancer Specimens

Robert K. Bright,[1] Cathy D. Vocke, Michael R. Emmert-Buck, Paul H. Duray, Diane Solomon, Patricia Fetsch, Johng S. Rhim, W. Marston Linehan, and Suzanne L. Topalian[2]

Surgery Branch [R. K. B., C. D. V., W. M. L., S. L. T.], Laboratory of Pathology [M. R. E-B., P. H. D., D. S., P. F.] and the Laboratory of Molecular Oncology [J. S. R.], National Cancer Institute, NIH, Bethesda, Maryland 20892

ABSTRACT

Difficulty in establishing long-term human prostate epithelial cell lines has impeded efforts to understand prostate tumorigenesis and to develop alternative therapies for prostate cancer. In the current study, we describe a method that was successful in generating 14 immortal benign or malignant prostate epithelial cell cultures from primary adenocarcinomas of the prostate resected from six successive patients. Immortalization with the E6 and E7 transforming proteins of human papilloma virus serotype 16 was necessary to establish long-term cultures. Microscopic examination of fresh tumor specimens exhibited a variable mixture of benign and malignant epithelium. Thus, single-cell cloning of tumor-derived cell cultures was essential for defining tumor cell lines. Efforts to characterize these cultures using traditional criteria such as karyotype, growth in nude mice, and prostate-specific antigen expression were noninformative. However, allelic loss of heterozygosity (LOH) represents a powerful alternative method for characterizing tumor cell lines originating from primary adenocarcinomas of the prostate. Microdissected fresh tumors from four of six patients revealed LOH at multiple loci on chromosome 8p, as assessed by PCR. LOH on chromosome 8p matching the patterns found in microdissected tumors was also observed in a tumor-derived cell line and its clones, as well as in one clone from a tumor-derived cell line from a second patient. LOH was not observed in immortal lines generated from autologous benign prostatic epithelium, seminal vesicle epithelium, or fibroblasts. The multifocal nature of prostate cancer, as well as the presence of an entire spectrum of malignant transformation within individual prostate glands, necessitates this type of careful analysis of derivative cell cultures for their validation as *in vitro* models that accurately reflect the primary cancers from which they are derived.

INTRODUCTION

In recent years, prostate cancer has emerged as the most commonly diagnosed cancer in men in the United States. In this year alone, new cases of prostate cancer are estimated to approach 300,000 with more than 40,000 deaths, resulting in a cancer mortality rate second only to lung cancer (1). Although prostate cancer mortality commonly results from metastatic disease, nearly 60% of newly diagnosed patients present with localized primary tumors. Surgery and radiation therapy are often effective in treating localized disease, but disseminated metastatic disease is largely untreatable. Despite considerable scientific effort, relatively little is known about the biological events causing the initiation and progression of prostate cancer. The development of new strategies for the treatment of adenocarcinoma of the prostate necessitates an increased understanding of the cellular and molecular events involved in the generation of primary prostate cancer and its metastatic progression.

Rodent models have provided valuable insights into the biology and pathology of primary prostate cancer, as well as useful systems for assessing novel treatment strategies *in vivo* (2). However, transferring information to the human disease situation has often proved difficult. Therefore, the generation of immortal human prostate epithelial cell cultures that accurately reflect the *in situ* characteristics of benign or malignant prostatic epithelium is imperative. To date, only four prostate cancer cell lines, initiated from metastatic lesions, have provided the basis for the majority of *in vitro* experiments concerning the biological and molecular events regulating prostate tumorigenesis. Extensive progress has been made toward the *in vitro* cultivation of short-term lines from primary (nonmetastatic) prostate cancers. These advances have included culture media development and improvements in fresh tissue preparation and prostate epithelial cell culture techniques (3, 4). However, the establishment and maintenance of long-term human prostate epithelial cell lines from primary tumors have been unsuccessful in the absence of *in vitro* immortalization. To this end, only a small number of reports describing long-term immortalized cell lines exist, and these have been limited to normal prostatic epithelial cultures (5–8). Thus, the goal of the current study was to develop reliable methods for generating and characterizing continuously proliferating prostate cancer cell lines derived from primary tumors.

Beyond the difficulties inherent in establishing immortal prostate epithelial cell lines are the problems associated with distinguishing cultivated prostate cancer from normal epithelial cells. Past cytogenetic evaluation of multiple, short-term prostate epithelial cell cultures has revealed that the majority of lines generated from localized prostate cancers exhibit a normal male karyotype (9–11). This, combined with the unremarkable microscopic morphology of short-term cultures and a pervasive lack of success with xenotransplantation, has rendered accurate identification and characterization of human primary prostate cancer cell lines extremely difficult.

The initiation of prostate cancer is believed to occur as a result of multiple genetic changes within the cell, including the inactivation of potential tumor suppressor genes as manifested by allelic chromosomal deletions (reviewed in Ref. 12). Early studies examining chromosomal deletions in fresh (noncultured) primary prostate cancer specimens exhibited allelic LOH[3] on chromosomes 8p, 10q, and 16q (13–15). Subsequent studies confirmed a remarkably high percentage of allelic loss on the short arm of chromosome 8, thus moving chromosome 8p to the forefront of the list of potential sites for prostate cancer-associated tumor suppressor genes (16–18). Moreover, recent examinations of 99 microdissected tumors (19) and 54 microdissected PIN lesions (20) for LOH on the short arm of chromosome 8p demonstrated strong evidence for the inactivation of a tumor suppressor gene(s) on chromosome 8p12-21 when compared with matched normal controls. Accordingly, examination of LOH within this minimal deletion region on chromosome 8p12-21 represents a potentially powerful alternative method for the identification and characterization of human prostate epithelial cell lines derived from primary tumors. In this study, we describe the successful generation and unique genetic characterization of multiple immortalized human tumor cell lines derived from primary adenocarcinomas of the prostate.

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[1] Present address: Karmanos Cancer Institute, 110 East Warren Avenue, Detroit, MI 48201-1359.

[2] To whom requests for reprints should be addressed, at the Surgery Branch, National Cancer Institute, NIH, 10/2B47, Bethesda, MD 20892.

[3] The abbreviations used are: LOH, loss of heterozygosity; PIN, prostatic intraepithelial neoplasia; FBS, fetal bovine serum; PSA, prostate-specific antigen; PAP, prostatic acid phosphatase; PBL, peripheral blood lymphocyte; BPH, benign prostatic hypertrophy.

MATERIALS AND METHODS

Initiation of Primary Cell Cultures. Tissue specimens used for generating cell lines were obtained from six consecutive patients undergoing radical prostatectomies at the National Cancer Institute for treatment of intermediate- to high-grade localized prostate cancer (Gleason grades 6–8, tumor stages T2C to T3C). Fresh prostatectomy specimens obtained directly from the operating room were dissected under sterile conditions by an experienced pathologist. Tissues designated as normal prostate, prostate cancer, or normal seminal vesicle on gross inspection were dissected separately for the purpose of generating cell cultures. Cultures were initiated by mechanical disruption (<1-cm diameter fragments) or enzymatic digestion (>1-cm fragments; Ref. 21). Specimens from patients 1510 and 1512 were prepared by enzymatic digestion, whereas other cultures were initiated by mechanical disruption. For enzymatic digestion, minced tissue was suspended in 100 ml of digestion medium and left on a stir plate overnight at room temperature. The resulting single-cell suspension was then washed with sterile PBS, resuspended in growth medium (see below), and dispensed into 6-well plates coated with type I rat tail collagen (Collaborative Biomedical Products, Bedford, MA). For mechanical disruption of specimens, tissue fragments were carefully minced into 2–3-mm cubes in a small volume of growth medium, and the resultant slurry of tissue and cells was dispensed into 6-well plates. All cultures were initiated in a volume of 1 ml per well and incubated at 37°C, 5% CO_2. They were not disturbed for 2–3 days to allow viable cells and tissue chunks to settle and attach to the plates. Then, the unattached debris was carefully aspirated, and wells were refed with 3–5 ml of fresh medium. Culture medium was routinely replaced every 2–4 days, and proliferating adherent cells were passaged after detachment with trypsin. Established growing cultures were maintained in tissue culture flasks (Falcon, Becton Dickinson, Lincoln Park, NJ). Growth medium for prostate and seminal vesicle epithelial cell lines consisted of keratinocyte serum-free medium (Keratinocyte-SFM, Life Technologies, Grand Island, NY) containing 25 μg/ml bovine pituitary extract, 5 ng/ml epidermal growth factor, 2 mM L-glutamine, 10 mM HEPES buffer, antibiotics, and 5% heat-inactivated FBS (Biofluids, Rockville, MD). For the initiation of epithelial cultures from fresh tissue specimens, the concentration of FBS was reduced to 1–2%, and/or cholera toxin (Sigma, St. Louis, MO) was added at 10–20 ng/ml to guard against outgrowth of contaminating fibroblasts. In the rare event that fibroblasts persisted in epithelial cell cultures, differential trypsinization (incubation for 1–2 min at 37°C, followed by washing away detached fibroblasts to leave the more adherent epithelial cells) was extremely successful in achieving pure epithelial cell cultures.

Autologous fibroblast cell lines were generated from mechanically disrupted benign prostate stromal tissue and cultured in RPMI 1640 containing 10% heat-inactivated FBS. Autologous EBV-transformed B-cell lines were generated using standard techniques and cultured in RPMI 1640 + 10% FBS.

Metastatic Prostate Cancer Cell Cultures. The adherent cell lines LNCaP, DU145, PC-3 (American Type Culture Collection, Rockville, MD), and TSU-Pr1 (kindly provided by Dr. William Isaacs, Johns Hopkins University, Baltimore, MD) were maintained in RPMI 1640 supplemented with 10% FBS.

Immortalization of Primary Cell Cultures. Cell culture immortalization was accomplished by transduction of actively proliferating cells with a recombinant retrovirus encoding the E6 and E7 transforming proteins of human papilloma virus serotype 16 and the eukaryotic selection marker neomycin phosphotransferase, designated LXSN16E6E7 (generously provided by Dr. Denise Galloway, Fred Hutchinson Cancer Research Center, Seattle, WA; Ref. 22). In preparation for immortalization, short-term epithelial cell cultures (culture passages 1–3) were split 1:2 and allowed to reattach in 6-well plates for at least 48 h, yielding cultures that were 50–60% confluent. Transduction with the LXSN16E6E7 retrovirus was accomplished by replacing the culture medium with culture supernatant collected from the retrovirus producer line PA317, in the presence of 10 μg/ml DEAE-dextran (Sigma), for a period of 24 h.

Single-Cell Cloning of Immortalized Cell Cultures. Clonal populations of immortal epithelial cell cultures were generated for use in LOH characterization studies. Briefly, confluent cell cultures were harvested with trypsin, washed, and counted. Cells were serially diluted to a concentration of 2–5 cells/ml in keratinocyte growth medium (see above) and dispensed into 8–10 individual 96-well flat-bottom microculture plates at 200 μl/well. Confluent wells originating from dilutions of <1 cell/well were expanded to 24-well plates to ensure enough cells for DNA extraction and cryopreservation.

Immunocytochemical Analysis. For immunocytochemical studies of immortalized cultured cells, cells were harvested with trypsin, washed, and pelleted. Cell pellets were subsequently fixed in 10% buffered formalin and embedded in paraffin. Fresh tissue sections from prostate specimens were also fixed in formalin and paraffin embedded. Five-μm sections were prepared from fresh tumor specimens or cultured cell blocks and mounted on charged slides (Fisher Scientific, Pittsburgh, PA; Ref. 23). Immunocytochemistry was performed using the avidin-biotin peroxidase complex method with the following primary antibodies: monoclonal or polyclonal antihuman PSA (DAKO Corp., Carpenteria, CA); polyclonal antihuman PAP (DAKO Corp.); antihuman cytokeratin CAM 5.2 (Becton-Dickinson, San Jose, CA); and antihuman cytokeratin AE1/AE3 (Boehringer-Mannheim, Indianapolis, IN). Cell lines and tumor tissue sections were evaluated based on the percentage of cells staining (<25%, 25–50%, 50–75%, or >75%), as well as staining intensity (1+ to 4+).

Flow Cytometry. For future studies and further characterization, it was of interest to determine the extent of expression of surface molecules of immunologic importance on the long-term prostate epithelial cell lines. Immortalized cell cultures were harvested and stained with the following monoclonal antibodies: CD54 (anti-ICAM-1), CD80 (anti-B7.1), CD86 (anti-B7.2; Becton-Dickinson), W6/32 (anti-HLA-A,B,C), and L243 (anti-HLA-DR) (American Type Culture Collection; Ref. 21). To enhance surface expression of MHC molecules, cells were cultured in the presence of IFN-γ 500 units/ml for 72 h before flow cytometric analysis.

Microdissection and DNA Extraction. Microdissection of selected foci of normal prostate epithelial cells or invasive tumor cells from frozen tissue sections was performed under direct light microscopic visualization as described previously (24–26). Briefly, unstained 5-μm histological tissue sections were prepared on glass slides. The adjacent section was stained with H&E. Specific cells of interest were selected from the eosin-stained slides and microdissected from the unstained slide using a disposable, modified 30-gauge needle. DNA was extracted from $1–5 \times 10^3$ cells procured by microdissecting regions less than 2 mm in diameter to minimize the potential effects of cellular heterogeneity. DNA was also extracted from $1–5 \times 10^4$ cells obtained from actively growing immortalized cultures. Cells were immediately resuspended in a solution (20 μl for microdissected cells or 200 μl for cultured cells) containing 0.01 M Tris-HCl (pH 8.0), 1 mM EDTA, 1% Tween 20, and 0.1 mg/ml proteinase K, and incubated overnight at 37°C. After incubation, the mixture was boiled for 5–10 min to inactivate the proteinase K and stored at 4°C for subsequent PCR analysis.

Detection of LOH. The polymorphic DNA markers studied for the detection of LOH on chromosome 8p12-21 included: *SFTP-2*, *D8S133*, *D8S136*, *NEFL*, *D8S137*, *D8S131*, *D8S339*, and *ANK*. PCR was performed as described previously (19). Briefly, 12.5-μl PCR reaction mixtures contained 200 μM dATP, dGTP, and dTTP; 40 μM dCTP; 0.8 mM primers (Research Genetics, Huntsville, AL, or synthesized on an Applied Biosystems DNA synthesizer); 2 μCi [α-^{32}P]dCTP; 16 μM tetramethylammonium chloride (27); 1× PCR reaction buffer (containing 1.5 mM $MgCl_2$); and 1 unit of *Taq* polymerase (Boehringer Mannheim). Five percent DMSO was added to reactions for the markers *D8S133* and *D8S137* to improve the amplification and resolution of the products. Reactions with all markers were performed as follows: 2 min at 95°C, followed by 28–40 cycles (depending on the marker) of annealing and extension (95°C for 30 s, annealing temperature for 30 s, and 72°C for 30 s) and a 2-min incubation at 72°C. Annealing temperatures for each marker were determined empirically after an initial estimate based on primer length and composition.

The labeled amplified DNA samples were denatured for 5–10 min at 90°C and loaded onto a gel consisting of 7% acrylamide (30:0.8 acrylamide:bisacrylamide), 5.6 M urea, 32% formamide, and 1× TBE (0.089 M Tris (pH 8.3), 0.089 M borate, and 0.002 M EDTA; Ref. 28). Samples were electrophoresed at 95 W for 2–4 h. Gels were then transferred to sequencing gel filter paper (Bio-Rad), and autoradiography was performed with Kodak X-OMAT film. The criterion for LOH was at least 75% loss of one allele compared with an autologous fresh PBL control, as determined by direct visualization by three independent investigators. When sufficient DNA was available, LOH was verified with at least two independent experiments.

RESULTS

Tissue Procurement for Cell Culture. Aware of the historical difficulties associated with generating immortal prostate cancer cell

Table 1 *Microscopic analysis of fresh prostate tissue specimens*

Patient no.	Tissue source[b]	% Total cells[a]			
		Benign	BPH	PIN	Tumor
1510	Tumor	40[c]	0	40[c]	60
1512	Normal prostate	100	0	0	0
	Tumor	90[c]	90[c]	0	10
1519	Normal prostate	100	0	0	0
	Tumor	50	0	0	50
1532	Normal prostate	95	0	5	0
	Tumor 1	100	0	0	0
	Tumor 2	0	0	0	100
1535	Normal prostate	20[d]	0	0	0
	Seminal vesicle	100	0	0	0
	Tumor 1	0	0	0	100
	Tumor 2	5	0	10	85
1542	Normal prostate	0	95	5	<1[e]
	Seminal vesicle	100	0	0	0
	Tumor 1	0	0	40	60
	Tumor 2	0	0	40	60
	Tumor 3	0	0	40	60

[a] Estimation from microscopic examination of 10–20 high-power fields.
[b] Grossly dissected by an experienced pathologist.
[c] A mixture of cell types.
[d] Eighty percent of specimen consisted of benign fibromuscular stroma.
[e] One microscopic focus of cancer noted.

lines from primary (nonmetastatic) specimens, we initially selected the largest grossly apparent tumor nodules (1–3 cm diameter) as the fresh tissue source for generating cultures. Subsequent microscopic analysis of the immediately adjacent tissue sections from the first three attempts (patients 1510, 1512, and 1519) revealed that "tumor" specimens actually contained a variable mixture of benign prostatic epithelium, BPH, PIN, and invasive tumor cells. However, "normal" specimens from patients 1512 and 1519 consisted entirely of benign prostatic epithelium (Table 1).

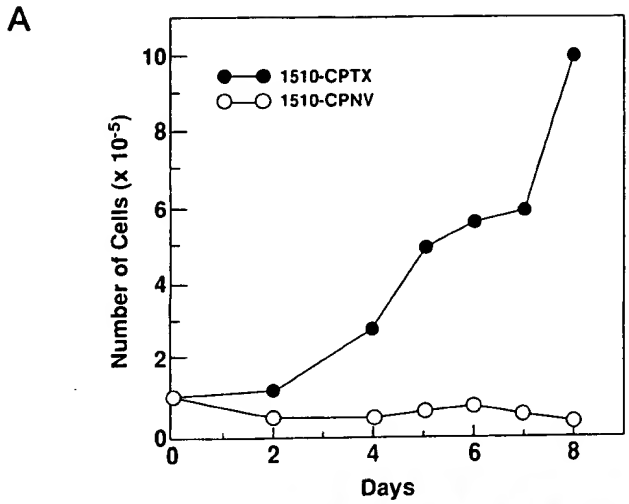
To increase the likelihood of obtaining pure tumor tissue for starting tumor cell lines from subsequent patients, smaller tissue fragments (<1 cm) were procured, with neighboring sections designated for tissue culture, and frozen and paraffin sections. In addition, whenever possible, multiple, distinct tumor tissue fragments were selected from individual specimens for culture initiation. By using these more stringent conditions, it was possible to obtain tissue sections containing at least 95% neoplastic cells (PIN plus invasive cancer) in six of seven attempts on three radical prostatectomy specimens (patients 1532, 1535, and 1542). In addition, tissue fragments suitable for initiating three benign prostate epithelial cell lines and two benign seminal vesicle epithelial cell lines were successfully dissected from these radical prostatectomy specimens (Table 1).

Immortalization and Immunocytochemical Characterization of Prostate-derived Cell Lines. All but 1 of the 17 tissue specimens listed in Table 1 (normal prostate from patient 1519) were readily established in short-term culture. However, cell proliferation was relatively slow, and *in vitro* immortalization of epithelial cell cultures was necessary to establish actively growing cultures capable of surviving beyond 5–6 weeks. Adherent monolayer cultures were transduced at the second or third passage with a recombinant retrovirus encoding the E6 and E7 transforming proteins of human papilloma virus serotype 16, resulting in the establishment of 16 long-term epithelial cell lines: 4 derived from normal prostate, 2 from seminal vesicle, and 10 from primary tumor specimens. In addition, immortal fibroblast lines initiated from prostatic stroma in four patients were established. Thus, 20 of 20 attempts to immortalize cell lines derived from a variety of tissue types were successful. Successful transduction was confirmed by cell survival in G418 at a concentration of 1 mg/ml and by extended cell viability and rapid proliferation beyond 50 culture passages when compared with nonimmortalized cells cultured in parallel (Fig. 1*A*). Microscopically, all immortalized prostate epithelial cell lines exhibited a similar morphology, whether derived from benign or malignant tissue; thus, culture morphology was not a useful criterion for distinguishing benign from malignant cells (Fig. 1*B*).

To confirm the epithelial and prostatic origins of the prostate-derived cell lines, immunocytochemistry was performed on cell blocks from actively growing immortalized cultures. Both high and low molecular weight cytokeratins were expressed by all of the epithelial cell lines initiated in our laboratory, including those derived from normal prostate, normal seminal vesicle, and prostate cancer specimens. More than 75% of cells stained with 4+ intensity, similar to staining observed with the established metastatic prostate cancer cell lines LNCaP, DU145, PC-3, and TSU-Pr1. Thus, the epithelial origin of these cultures was confirmed. No significant cytokeratin expression was observed in control fibroblast lines or melanoma cells (data not shown).

Although positive cytokeratin expression indicated that cell lines generated from primary prostate cancer specimens were in fact epithelial in origin, it was also of interest to assess expression of the prostate-associated proteins PSA and PAP by these cultures. Only the immortalized prostate tumor-derived cell line generated from patient 1519 (1519-CPTX) expressed detectable levels of these proteins (>75% of cells staining with 2–3+ intensity, and >75% with 4+ intensity, respectively) after five culture passages. However, after 30 culture passages, expression of PSA and PAP was no longer detectable in 1519-CPTX. Furthermore, expression was not inducible in late passages of this cell line by IFN-γ, 5-aza-2′-deoxycytidine, or dihydroxytestosterone (data not shown). Immunohistochemical examination of fixed prostate cancer tissue sections for the expression of PSA and PAP often showed weak and heterogeneous staining of tumor cells, with some tumor foci demonstrating no detectable expression of these proteins. In contrast, all normal glands in the same microscopic sections stained strongly and uniformly for PSA and PAP (Fig. 2). The sometimes weak, heterogeneous expression of PSA and PAP by prostate cancer cells *in situ* may explain the absence of expression in our immortalized prostate tumor-derived cell lines. However, lack of expression in our benign prostate epithelial cell lines does not correlate with the strong expression observed in the corresponding tissue sections, indicating that loss of PSA and PAP expression may also occur as a consequence of *in vitro* cell culture.

Examination of Chromosome 8p for LOH in Microdissected Tissues. As noted above, our "prostate cancer" cell lines were in most cases actually derived from tissue samples containing a mixture of benign and malignant cell types (Table 1). Because all cultures required retroviral transformation to induce long-term proliferation, and because benign and malignant transformed prostatic epithelial cells were indistinguishable on morphological and histochemical grounds, we investigated the use of LOH analysis as an alternative means of characterizing our newly established cultures. To direct our studies of cultured cell lines, LOH on chromosome 8p12-21 was first assessed in microdissected foci of tumor or normal epithelial cells from the corresponding fresh tissue sections. A panel of eight microsatellite markers, shown previously to detect a high percentage of LOH in microdissected prostate cancer specimens (19), was selected to identify chromosome 8p deletions. Hypothesizing that microscopically normal-appearing cells might contain LOH as a precursor to malignant transformation, we used cryopreserved fresh autologous PBLs as the normal controls for LOH analysis. All six patients proved to be heterozygous (informative) at four or more of the eight loci examined upon analysis of DNA from fresh PBLs. However, for two patients (1519 and 1532), microdissected tumor specimens did not yield evidence of LOH, indicating that LOH analysis might not be useful in characterizing cell cultures derived from those specimens (Table 2). In contrast, microdissected tumors from patients 1510 and 1512 demonstrated LOH at all examined informative loci. For patient 1535, six



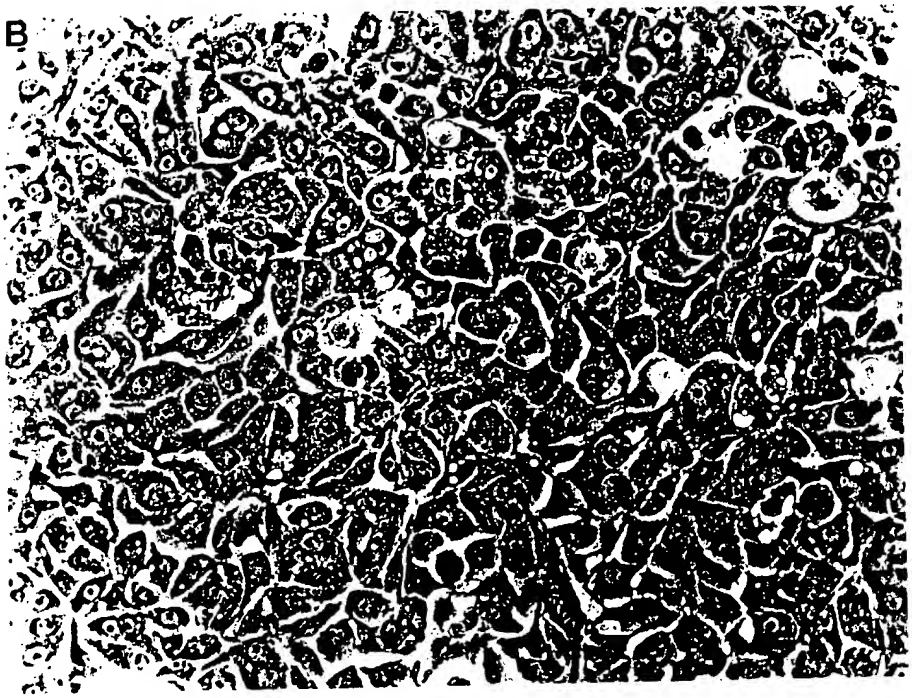


Fig. 1. Morphological and growth characteristics of an immortalized prostate epithelial cell line. *A*, immortalization with the retrovirus LXSN16E6E7 was necessary to achieve continued proliferation of culture 1510-CP, initiated from a prostate cancer specimen. Cells were transduced (*1510-CPTX*) or not (*1510-CPNV*) at culture passage 3, and proliferation in 24-well plates was monitored at passages 10 and 5, respectively. *B*, photomicrograph of 1510-CPTX after 10 culture passages (×200, phase contrast). This culture appearance is typical of other prostate epithelial cell lines generated from benign or malignant specimens.

distinct microdissected foci of tumor were examined, and all exhibited similar patterns of LOH. Of interest, LOH analysis of 12 distinct microdissected tumors from patient 1542 revealed different patterns of LOH, with 3 of 12 exhibiting retention of all four alleles examined (Table 3). Microdissected normal epithelium failed to show evidence of LOH on chromosome 8p, with the exception of specimens derived from patient 1510. All three normal microdissected foci from patient 1510 exhibited extensive LOH consistent with the pattern of LOH observed in the autologous tumor, emphasizing the importance of using PBLs as the normal controls for this type of study.

LOH Analysis of Immortalized Cell Lines from Patient 1542. LOH in cell cultures generated from patient 1542 was of special interest in light of the diverse patterns of LOH manifested in 12 distinct microdissected tumor foci. This patient was informative at *D8S133*, *D8S136*, *D8S137*, *D8S131*, *D8S339*, and *ANK*. Four of those loci were closely examined for allelic loss in cultures derived from tumor, normal prostate, normal seminal vesicle, and normal fibroblasts (Table 3). Repeated analysis of early passage bulk cultures (passages 3, 6, and 13) derived from tumor, designated 1542-CP_3TX, failed to reveal LOH for any of the four microsatellite markers examined. However, after 21 serial culture passages (approximately 5 months), 1542-CP_3TX exhibited loss of the upper allele at all four loci examined. This pattern of loss was identical to that found in microdissected tumor focus 7. Thirty single-cell clones were generated from passage 23 of 1542-CP_3TX, and all demonstrated a pattern of LOH identical to that of the uncloned late-passage culture and microdissected tumor 7, suggesting the clonal or near-clonal composition of the bulk late-passage cell line. These findings also suggested that the failure to detect LOH in early passages of 1542-CP_3TX might reflect the presence of multiple tumor clones in the bulk culture with different patterns of LOH, which would preclude the detection of LOH with a PCR-based technique. To investigate this, single-cell clones were generated from an early passage (passage 8) of 1542-CP_3TX and examined for LOH (Fig. 3). Seven of nine clones did not manifest LOH at *D8S136* or *D8S131*, similar to 3 of 12 microdissected tumors from patient 1542.

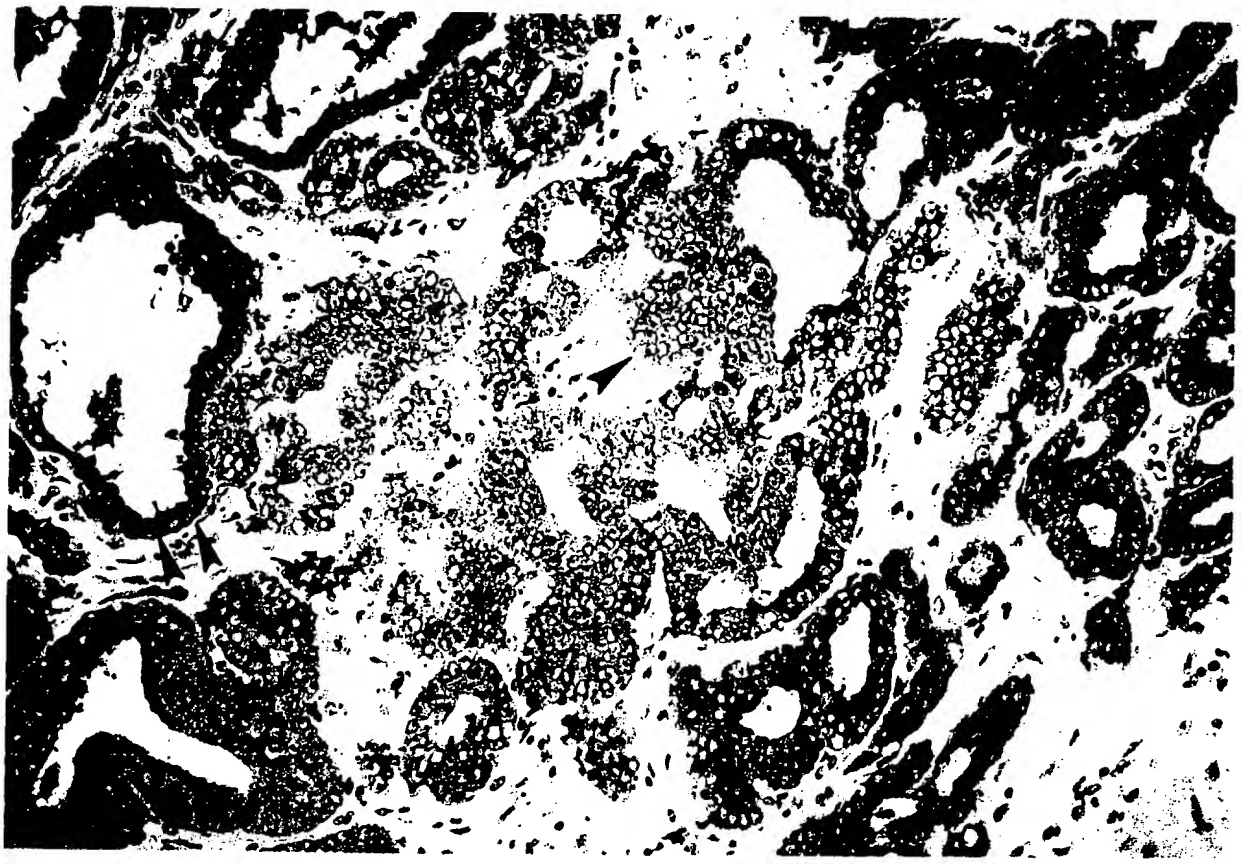


Fig. 2. Expression of PSA by benign and malignant prostate epithelial cells *in situ*. A paraffin-embedded tissue section from the radical prostatectomy specimen from patient 1510 contains areas of invasive prostate cancer (*single arrow*), as well as normal prostatic epithelium (*double arrows*). *Brownish* pigmentation indicates binding of an anti-PSA monoclonal antibody. PSA expression by normal prostatic epithelial cells is intense and homogeneous, whereas expression by cancer cells is weak and heterogeneous. Intervening stromal cells do not express PSA. (×200).

However, a single clone (clone 4) exhibited a pattern of LOH similar to that of microdissected tumor 7, the late passage of 1542-CP_3TX and its derivative clones, indicating that the tumor clone(s) that dominated the late-passage bulk culture apparently resided in very early culture passages. Of interest, clone 1 from the early passage 1542-CP_3TX exhibited a different pattern of LOH than that observed for the other eight early passage clones, with loss of the lower alleles of *D8S133*, *D8S136*, and *D8S131*. This was again consistent with the pattern of LOH detected in two microdissected tumors (1 and 3). It is important to note that LOH was not detected in repeated experiments with early and late passages of immortalized cultured normal prostatic epithelium, seminal vesicle, or fibroblasts from patient 1542, arguing against the possibility that the LOH observed in cells derived from tumor might represent a culture artifact.

Examination of LOH of Chromosome 8p12–21 in Cell Cultures Derived from the Five Remaining Patients. In patients 1510 and 1512, LOH was detected at multiple loci in microdissected tumor specimens (Table 2). In contrast, immortalized epithelial cultures generated from corresponding cancer-containing tissue specimens

Table 2 *LOH on chromosome 8p in microdissected foci of prostate cancer or benign epithelium*

Patient	No. of foci tested	Chromosome 8p locus							
		SFTP-2[a]	*D8S133*	*D8S136*	*NEFL*	*D8S137*[b]	*D8S131*	*D8S339*	*ANK*
1510									
Tumor	2	●	●	●	NI	●	NI	●	●
Normal	3	●	●	●	NI	●	NI	●	●
1512									
Tumor	1	NI	●	●	●	ND	NI	●	ND
Normal	1	NI	○	○	○	ND	NI	○	ND
1519									
Tumor	1	NI	○	○	○	NI	○	○	○
Normal	1	NI	○	○	○	NI	○	○	○
1532									
Tumor	8	NI	○	○	○	ND	NI	○	ND
Normal	1	NI	○	○	○	ND	NI	○	ND
1535									
Tumor	6	●	●	●	●	NI	●	○	NI
Normal	1	○	○	○	○	NI	○	○	NI

[a] ●, LOH; NI, not informative (homozygous alleles); ○, retention of heterozygosity.
[b] ND, not determined.

Table 3 *LOH on chromosome 8p in microdissected prostate tissues and immortalized cell lines from patient 1542*

Cell source	Chromosome 8p locus			
	D8S133[a]	*D8S136*	*D8S137*	*D8S131*
Microdissected foci				
Normal epithelium	NL	NL	NL	NL
Tumor				
1	LL	LL	LL	LL
2	ND	ND	NL	NL
3	LL	LL	LL	LL
4	NL	NL	NL	NL
5	LL	LL	NL	NL
6	NL	NL	NL	NL
7	LU	LU	LU	LU
8	NL	NL	NL	NL
9	LU	ND	NL	NL
10	LL	LL	NL	NL
11	LU	LL	NL	NL
12	NL	LL	LL	NL
Cultured cell lines				
NPTX (p20)[b]	NL	NL	NL	NL
CP_3TX (p3,6,13)	NL	NL	NL	NL
CP_3TX				
clone 1 (p8)	LL	LL	ND	LL
clone 3[c] (p8)	ND	NL	ND	NL
clone 4 (p8)	LU	LU	ND	LU
CP_3TX (p21)	LU	LU	LU	LU
CP_3TX clones[d] (p23)	LU	LU	LU	LU

[a] NL, no LOH; LL, loss of the lower allele; ND, not determined; LU, loss of the upper allele.
[b] Number of sequential culture passages.
[c] Representative of seven individual clones.
[d] Representative of 30 individual clones.

failed to manifest LOH when examined on a bulk level at early or late culture passages (data not shown). Likewise, clones grown from late culture passages (passage 23 for 1510-CPTX, passage 31 for 1512-CPTX) failed to show evidence of LOH. This may reflect the presence of significant amounts of normal prostatic epithelium in the tissue specimens from which these cultures were generated (Table 1), with overgrowth of normal cells *in vitro*. Cloning these cell lines at very early culture passages may yield more rewarding results.

Examination of microdissected tumor foci from patients 1519 (one focus) and 1532 (eight foci) failed to reveal LOH (Table 2). Nevertheless, cultures established from these tumors were assessed for LOH. In the case of patient 1519, examination of the bulk culture

Fig. 3. PCR analysis of microsatellite D8S136 on fresh and cultured cells from patient 1542. *Lane 1*, 1542-NPTX, passage 26. *Lane 2*, microdissected tumor 11. *Lane 3*, uncloned 1542-CP_3TX, passage 21. *Lanes 4–6*, tumor clones 1, 3, and 4 derived from the 8th passage of 1542-CP_3TX.

1519-CPTX showed retention of heterozygosity at six informative loci that were examined. However, among 11 single-cell clones derived from culture passage 24, 1 showed LOH at a single locus, *D8S133*. In the case of patient 1532, the bulk-cultured line 1532-CP_2TX, generated from one of two tumor specimens procured (Table 1), showed LOH at *D8S133*, *D8S136*, and *NEFL*, but only after prolonged culture (passage 24). All 10 clones generated from the late culture passage also showed the same pattern of loss (data not shown). However, an immortalized culture derived from normal prostate tissue from patient 1532 failed to show evidence of LOH even after 20 culture passages. Likewise, an autologous immortalized fibroblast line retained heterozygosity at the same three alleles that were lost in 1532-CP_2TX. Thus, although we cannot exclude the possibility that the LOH observed in a single 1519-CPTX clone and in 1532-CP_2TX was induced by *in vitro* culture conditions, our experience with cultures derived from patient 1542 (see above) suggests that these findings may reflect LOH existing in an *in situ* tumor focus that was not dissected for analysis.

Interesting results were obtained with cultures derived from patient 1535. In this case, extensive LOH was documented in six separate microdissected tumor foci, all showing the same pattern of loss (Table 2). Early and late-passage cultures generated from prostate cancer, as well as from normal prostate and normal seminal vesicle, failed to show LOH. Likewise, 11 tumor clones generated at culture passage 27 failed to show loss. However, cloning of an early passage tumor culture (passage 14) revealed one clone with a pattern of LOH matching that of the six microdissected tumor foci. These results reinforce those observed with patient 1542 and argue that early cloning of immortalized cultures generated from histologically heterogeneous prostate cancer specimens may be needed to obtain pure tumor cultures.

Expression of MHC Molecules by Immortalized Cell Lines Derived from Prostate Cancer. Examination of surface MHC expression on immortalized tumor-derived cell lines was of importance in considering the potential usefulness of these lines for immunological studies. Cultures derived from all six patients expressed signifi-

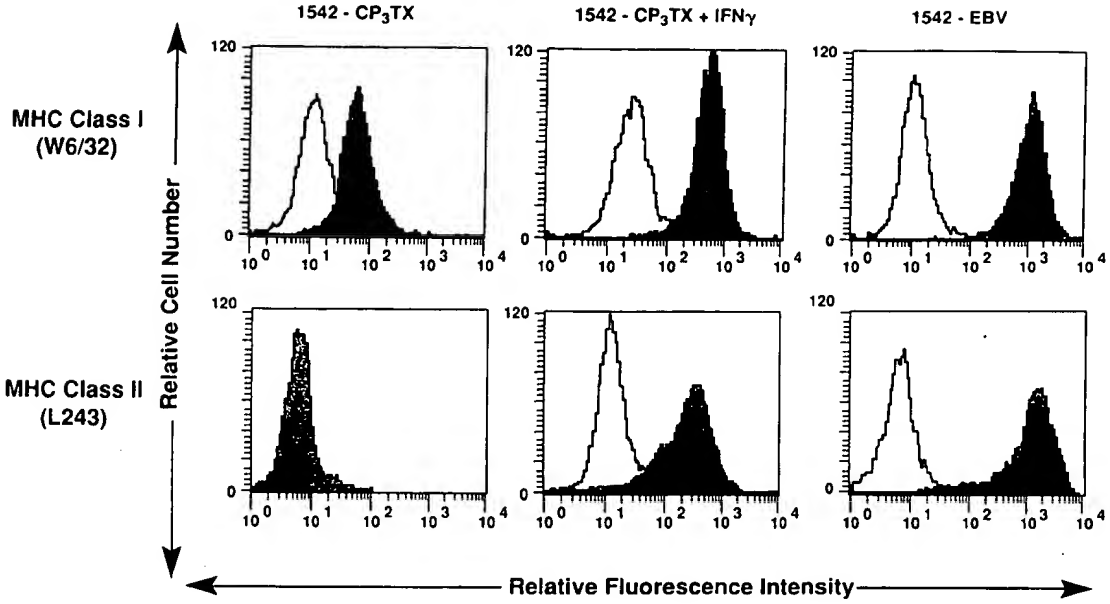


Fig. 4. IFN-γ induces enhanced surface expression of MHC class I and II molecules on 1542-CP_3TX. Untreated 1542-CP_3TX cells expressed a moderate amount of class I molecules (staining with monoclonal antibody W6/32) but did not express detectable amounts of class II molecules (monoclonal antibody L243). After exposure to IFN-γ 500 units/ml for 3 days, class I expression was enhanced, and class II expression was induced. MHC expression by autologous EBV-transformed B cells is shown for comparison.

cant surface levels of MHC class I and the adhesion molecule ICAM-1 as determined by flow cytometry. None of the immortalized lines expressed detectable levels of either MHC class II or the B7 family of costimulatory molecules (B7.1 and B7.2; data not shown). However, it was of interest to determine whether the expression of MHC molecules could be up-regulated in the presence of IFN-γ, as has been reported previously for melanoma cell lines (29). Immortalized tumor-derived cell lines 1532-CP_2TX, 1535-CP_1TX, and 1542-CP_3TX were cultured in the presence of 500 units/ml IFN-γ for 72 h and then assessed for MHC expression. All were induced to express significant amounts of MHC class II molecules. In addition, MHC class I molecule expression was enhanced when compared with untreated controls (Fig. 4). In this light, these immortalized tumor-derived cell lines represent potentially valuable reagents for studying $CD4^+$ and $CD8^+$ cell-mediated immune responses in patients with primary adenocarcinoma of the prostate.

DISCUSSION

The need for human prostate epithelial cell cultures as *in vitro* models to better understand the conditions affecting the initiation and growth of prostate cancer has long been recognized (30). Efforts spanning a half a century, since the pioneering work of Burrows *et al.*, have produced only a handful of cell lines derived from normal prostatic epithelium (5, 7). To date, only four readily available and commonly studied long-term human prostate carcinoma cell lines exist: DU145, PC-3, LNCaP, and TSU-Pr1. All four were isolated from metastatic lesions, thus leaving a void in reagents representing long-term human cell lines derived from primary localized adenocarcinoma of the prostate. The present study represents a focused effort to generate immortal cell lines from benign and malignant epithelial cells from fresh prostatectomy specimens and illustrates some of the well-chronicled difficulties involved. Although an experienced pathologist exercised extreme care to dissect pure tumor samples from fresh specimens, even the smallest tumor samples obtained for culture initiation contained mixtures of diverse cell types (stroma, normal epithelium, BPH, PIN, and/or invasive tumor). This is not surprising considering the tissue architecture of the peripheral zone of the prostate and the disseminated and multifocal character of primary prostate cancer. The unremarkable microscopic morphology and comparable growth rates of newly initiated normal and tumor cultures rendered mechanical separation and growth selection impossible. To our knowledge, primary prostate cancer cells do not continue to proliferate beyond 12 weeks in culture, and our cultures ceased to grow well before that. Therefore, immortalization with the E6 and E7 transforming proteins was necessary to establish actively growing long-term cultures. The 14 transformed prostate epithelial cultures derived from six patients in this study have been continuously passaged for more than 1 year. Such stable, long-term cultures are mandatory for molecular and immunological studies requiring a continual source of large numbers of cells. Although the transformation procedure may affect other aspects of cell phenotype, the availability of paired autologous cell lines derived from normal and malignant prostate epithelium, as well as fibroblasts and seminal vesicle epithelium, provides the opportunity for controlled studies.

Equally as challenging as establishing prostate epithelial cell lines is the need to characterize them. The tumor-derived cell lines in this study were confirmed as being of epithelial origin by their cytokeratin expression. In the absence of other epithelial cell types within the prostate gland, they therefore represented prostatic epithelium. However, only one line, 1519-CPTX, expressed PSA and PAP as assessed by immunocytochemistry, and it lost expression after prolonged continued culture passage. There is only one reported prostate cancer cell line that expresses PSA in culture, the metastasis-derived LNCaP (31). Studies examining PSA expression in LNCaP suggest that PSA expression is androgen responsive (32), but our efforts to up-regulate PSA in 1519-CPTX with androgen were unsuccessful. Typical hallmarks of malignant cell lines such as growth in nude mice or aneuploid karyotypes were unrevealing in this study (data not shown), as they have been in others (9). Subcutaneous inoculations of 1×10^7 cultured cells into nude mice failed to produce measurable tumors from 1510-CPTX, 1512-CPTX, or 1519-CPTX even after 4 months of observation, whereas inoculations of DU145 or the metastatic mela-

noma line 397-mel grew within 6 weeks. In addition, karyotypic analysis of these three primary prostate cancer-derived cell lines using standard banding techniques showed that most chromosome counts were within the normal male diploid range. Thus, there was a critical need to develop new methods for characterizing cell cultures derived from prostate cancer specimens.

LOH represents a powerful alternative method for characterizing tumor cell lines originating from primary adenocarcinomas of the prostate. The complex mixture of normal and malignant cells surrounding a tumor focus, along with the evident multiclonal nature of prostate cancer (12), has impaired the ability of techniques such as RFLP to detect LOH in prostate cancer. However, innovative microdissection techniques coupled with PCR technology have permitted more precise evaluation of LOH, revealing that >85% of prostate cancer foci exhibit LOH on chromosome 8p (19, 20). Microdissected tumors from four of the six patients described here revealed LOH at multiple loci on chromosome 8p12-21, and those patterns of loss were used as a basis for evaluating cell lines generated from neighboring tissue fragments. However, even this method of evaluation carries inherent uncertainties. For instance, in the case of patient 1542, microdissected counterparts to LOH patterns observed in cultured cell lines were encountered only with extensive sampling of multiple fresh tumor foci. In addition, cell lines exhibiting retention of the tested alleles cannot be evaluated for malignancy with this method, since microdissected tumors, as well as normal glands, may fail to show LOH. Conversely, allelic retention is not always characteristic of normal prostate epithelium, as shown in the unusual case of patient 1510, in whom multiple normal glands exhibited extensive LOH. Despite these methodological uncertainties, it was possible to characterize 1542-CP_3TX and its clones, as well as one clone from 1535-CP_1TX, as representing prostate cancer cell lines. In this study, single-cell cloning of cancer-derived cell lines represents a successful and perhaps necessary step toward defining tumor cultures in the setting of a heterogeneous starting cell population.

Efforts to generate defined, immortalized cell cultures from both malignant and normal prostate epithelial cells are critical to ongoing studies into the pathogenesis of prostate cancer. Such reagents are essential to the biological and genetic studies that will accelerate the development of new forms of prevention and therapy for this disease.

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Biosafety Level:	1	**Shipped:**	frozen
Medium & Serum:	See Propagation	**Growth Properties:**	adherent, single cells and loosely attached clusters
Organism:	*Homo sapiens* (human)	**Morphology:**	epithelial PHOTO
Tissue:	prostate; metastatic site: left supraclavicular lymph node carcinoma		
Cellular Products:	human prostatic acid phosphatase; prostate specific antigen [21889]		
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Comments:	**LNCaP** clone FGC was isolated in 1977 by J.S. Horoszewicz, et al., from a needle aspiration biopsy of the left supraclavicular lymph node of a 50-year-old Caucasian male (blood type B+) with confirmed diagnosis of metastatic prostate carcinoma. [21889] These cells are responsive to 5-alpha-dihydrotestosterone (growth modulation and acid phosphatase production). [23045] The cells do not produce a uniform monolayer, but grow in clusters which should be broken apart by repeated pipetting when subcultures are prepared. They attach only lightly to the substrate, do not become confluent and rapidly acidify the medium. Growth is very slow. The cells should be allowed to incubate undisturbed for the first 48 hours after subculture.

	When flask cultures are shipped, the majority of the cells become detached from the flask and float in the medium. Upon receipt, incubate the flask (in the usual position for monolayer cultures) for 24 to 48 hours to allow the cells to re-attach. The medium can then be removed and replaced with fresh medium. If desired, the contents of the flask can be collected, centrifuged at 300 X g for 15 minutes, resuspended in 10 ml of medium and dispensed into a single flask.
Receptors:	androgen receptor, positive; estrogen receptor, positive [23045]
Tumorigenic:	Yes, in soft agar Yes, the cells are tumorigenic in nude mice
Cytogenetic Analysis:	This is a hypotetraploid human cell line. The modal chromosome number was 84, occurring in 22% of cells. However, cells with chromosome counts of 86 (20%) and 87 (18%) also occurred at high frequencies. The rate of cells with higher ploidies was 6.0%.
Age:	50 years adult
Gender:	male
Ethnicity:	Caucasian
Propagation:	**ATCC complete growth medium:** RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate, 90%; fetal bovine serum, 10% **Temperature:** 37.0 C **Atmosphere:** air, 95%; carbon dioxide (CO2), 5%
Subculturing:	**Protocol:** 1. Remove and discard culture medium. 2. Briefly rinse the cell layer with 0.25% (w/v) Trypsin- 0.53 mM EDTA solution to remove all traces of serum that contains trypsin inhibitor. 3. Add 2.0 to 3.0 ml of Trypsin-EDTA solution to flask and observe cells under an inverted microscope until cell layer is dispersed (usually within 5 to 15 minutes). Note: To avoid clumping do not agitate the cells by hitting or shaking the flask while waiting for the cells to detach. Cells that are difficult to detach may be placed at 37°C to facilitate dispersal. 4. Add 6.0 to 8.0 ml of complete growth medium and aspirate cells by gently pipetting. 5. Add appropriate aliquots of the cell suspension to new culture vessels. Maintain cultures at a cell concentration between 1 X 10(4) and 2 X 10(5) cells/cm2. 6. Incubate cultures at 37°C. **Subcultivation Ratio:** A subcultivation ratio of 1:3 to 1:6 is recommended **Medium Renewal:** Twice per week
Freeze Medium:	Complete growth medium supplemented with 5% (v/v) DMSO **Storage temperature:** liquid nitrogen vapor phase
Doubling Time:	about 34 hours
Related Products:	Recommended medium (without the additional supplements or serum described under ATCC Medium) - ATCC 30-2001 recommended serum - ATCC 30-2020 purified DNA - ATCC CRL-1740D
References:	21889: Murphy G, editor. Models for prostate cancer. 37. New York: Liss; 1980, pp. 115-132. 22410: Gibas Z , et al. A high-resolution study of chromosome changes in a human prostatic carcinoma cell line (LNCaP). Cancer Genet. Cytogenet. 11: 399-404, 1984. PubMed: 6584201 23045: Horoszewicz JS , et al. **LNCaP** model of human prostatic carcinoma. Cancer Res. 43: 1809-1818, 1983. PubMed: 6831420 32283: Hu SX , et al. Development of an adenovirus vector with tetracycline-regulatable human tumor necrosis factor alpha gene expression. Cancer Res. 57: 3339-3343, 1997. PubMed: 9269991 33090: Boffa LC , et al. Invasion of the CAG triplet repeats by a complementary peptide nucleic acid inhibits transcription of the androgen receptor and TATA-binding protein genes and correlates with refolding of an active nucleosome containing a unique AR gene sequence. J. Biol. Chem. 271: 13228-13233, 1996. PubMed: 8662737

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Cancer Research

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Molecular Biology and Genetics

MHC Class I-restricted Recognition of a Melanoma Antigen by a Human CD4$^+$ Tumor Infiltrating Lymphocyte

Michael I. Nishimura[1], Dody Avichezer, Mary C. Custer, Catherine S. Lee, Christine Chen, Maria R. Parkhurst, Rochelle A. Diamond, Paul F. Robbins, Douglas J. Schwartzentruber and Steven A. Rosenberg

Surgery Branch, National Cancer Institute, Bethesda, Maryland 20892 [M. I. N., D. A., M. C. C., C. S. L., C. C., M. R. P., P. F. R., D. J. S., S. A. R.], and Division of Biology, California Institute of Technology, Pasadena, California 91125 [R. A. D.]

ABSTRACT

It is generally considered that MHC class I-restricted antigens are recognized by CD8$^+$ T cells, whereas MHC class II-restricted antigens are recognized by CD4$^+$ T cells. In the present study, we report an MHC class I-restricted CD4$^+$ T cell isolated from the tumor infiltrating lymphocytes (TILs) of a patient with metastatic melanoma. TIL 1383 I recognized HLA-A2$^+$ melanoma cell lines but not autologous transformed B cells or fibroblasts. The antigen recognized by TIL 1383 I was tyrosinase, and the epitope was the 368–376 peptide. Antibody blocking assays confirmed that TIL 1383 I was MHC class I restricted, and the CD4 and CD8 coreceptors did not contribute significantly to antigen recognition. TIL 1383 I was weakly cytolytic and secreted cytokines in a pattern consistent with it being a T_{h1} cell. The avidity of TIL 1383 I for peptide pulsed targets is 10–100-fold lower than most melanoma-reactive CD8$^+$ T cell clones. These CD4$^+$ T cells may represent a relatively rare population of T cells that express a T-cell receptor capable of cross-reacting with an MHC class I/peptide complex with sufficient affinity to allow

triggering in the absence of the CD4 coreceptor.

INTRODUCTION

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T cells can mediate the recognition and elimination of virus-infected cells, tissue allografts, cells expressing normal self antigens, and tumor cells in humans and in animal models. Mature T cells develop in the thymus from bone marrow-derived progenitor cells (reviewed in Refs. 1 and 2). T-cell antigens are recognized in association with MHC molecules, resulting in MHC-restricted antigen recognition (3) . T cells expressing the CD8 coreceptor on their cell surface recognize 8–10 amino acid peptide fragments bound to MHC class I molecules and thus are MHC class I restricted (4 , 5) . $CD8^+$ T cells are generally CTLs and commonly represent the effector arm of T cell-mediated immunity. T cells expressing the CD4 coreceptor on their cell surface recognize 10–15 amino acid peptide fragments bound to MHC class II molecules and thus are MHC class II restricted (4 , 5) . $CD4^+$ T cells are generally helper T cells that secrete cytokines that initiate and augment the function of CTL and B cells.

Broad cross-reactivity exists among T cells in the periphery and in the thymus, suggesting that epitope mimicry plays an important part in shaping the T-cell repertoire (6 , 7) . The sequence and relative abundance of individual peptides presented by the thymic epithelium can have dramatic effects on T-cell development (8 , 9) . Individual peptides are capable of positively selecting a diverse T-cell repertoire in fetal thymic organ cultures (10) . In the periphery, individual T-cell clones can cross-react with alloantigens (11) and a broad spectrum of peptides in the context of self MHC (12, 13, 14) . These studies suggest that cross-reactivity is an important mechanism for generating and maintaining T-cell immunity.

In the present study, we describe an HLA-A2-restricted, melanoma-reactive $CD4^+$ TIL^2 culture from melanoma patient 1383. Transient transfection experiments identified the antigen that was recognized by TIL 1383 I as tyrosinase. The epitope recognized by TIL 1383 I was a 9 amino acid peptide corresponding to amino acid residues 368–376 from tyrosinase. TIL 1383 I was weakly cytolytic and secreted cytokines in a pattern consistent with T helper 1 (T_{h1}) cells that produce IFN-γ, IL-2, GM-CSF, and TNF-α but not IL-4 or IL-10. The MHC class I restricted antigen recognition by $CD4^+$ T cells supports the notion that epitope mimicry exists in the immune response by T cells.

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Tumor Cell Lines.

All melanoma, immortalized fibroblasts (E6E7 transformed) and EBV B-cell lines used in this study were established from surgical specimens (TILs, melanoma, and fibroblasts) or peripheral blood mononuclear cells (EBV B) from melanoma patients undergoing

immunotherapy in the Surgery Branch, National Cancer Institute, as described previously (15, 16, 17) . Prostate cancer lines 1542 CP3TX (HLA-A2$^-$) and 1550 CPTX (HLA-A2$^+$) were kindly provided by Dr. Suzanne L. Topalian (Surgery Branch, National Cancer Institute, NIH, Bethesda, MD) and were established from patients undergoing radical prostatectomies at the National Cancer Institute as described previously (17) . Renal cell carcinoma lines UOK 181 (HLA-A2$^-$) and UOK 131 (HLA-A2$^+$) were kindly provided by Dr. W. Marston Linnehan (Urology Branch, National Cancer Institute, NIH, Bethesda, MD) and were established from patients undergoing radical nephrectomy at the National Cancer Institute as described previously (18) . Esophageal carcinoma cell lines HCE-4 (HLA-A2$^-$) and SKGT-5 (HLA-A2$^+$) were kindly provided by Dr. David S. Schrump (Surgery Branch, National Cancer Institute; Ref. 19). Colon carcinoma cell lines LOVO (HLA-A2$^-$) and SW480 (HLA-A2$^+$) lines, breast carcinoma cell lines MCF7 (HLA-A2$^+$) and MDA-MB-231 (HLA-A2$^+$), and ovarian carcinoma cell line SKOV3 (HLA-A2$^-$) were obtained from American Type Culture Collection (Rockville, MD). 624–28 MEL (HLA-A2$^-$) and 624–38 MEL (HLA-A2$^+$) were obtained by limiting dilution cloning of 624 MEL (20) . 888 A2 MEL, 397 A2 MEL, and SKOV3 A2 were obtained by transfecting 888 MEL, 397 MEL, and SKOV3, respectively, with a cDNA encoding HLA-A2 that was cloned into the pcDNA 3 eukaryotic expression vector (In Vitrogen, Carlsbad, CA). COS-7 cells that were used for transient transfection assays and T2 cells that were used for peptide recognition assays have been described elsewhere (21 , 22) . All tumor cell lines except the prostate cell lines were maintained in CM that consisted of RPMI 1640 (Biofluids, Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc., Rockville, MD) and 1% penicillin-streptomycin-glutamine (Life Technologies). The prostate cell lines were maintained in Keratinocyte-SFM (Life Technologies) supplemented with 5% heat-inactivated fetal bovine serum, 1% penicillin-streptomycin-glutamine, 10 mM HEPES (Biofluids, Rockville, MD), and 25 mM bovine pituitary extract (Life Technologies).

Generation of TIL Lines.

All TILs were established from surgical specimens obtained from patients with metastatic melanoma as described (15) . Viable cells (1 x 10^6; tumor and lymphocytes) from patient 1383 were plated in each of 12 wells of a 24-well plate in a total of 2 ml of TIL medium that consisted of AIM V medium (Life Technologies) supplemented with 5% heat-inactivated pooled AB human serum (Sigma Chemical Co., St. Louis, MO), 1% penicillin-streptomycin-glutamine, and 6000 IU/ml recombinant human IL-2 (Chiron, Emeryville, CA). All TIL cultures were expanded and maintained at densities between 0.5–2.0 x 10^6 cells/ml. TIL 1235 and TIL 1520 are HLA-A2 restricted CD8$^+$ TIL cultures that recognize the MART-1:27–35 and gp100:209–217 peptide epitopes, respectively (22 , 23) . TIL 888 is an HLA-A24-restricted CD8$^+$ TIL culture that recognizes tyrosinase (24) . TIL 1558 is an HLA-DR1-restricted CD4$^+$ TIL culture that recognizes a mutated triosephosphate isomerase that is expressed only by its autologous 1558 MEL (25) .

Plasmids.

The cloning of the HLA-A2.1 (22) , MART-1 (22) , gp100 (26) , tyrosinase (24) , TRP-2 (27) , and NY Eso-1 (28) cDNAs has been described elsewhere. All cDNAs were subcloned into the eukaryotic expression vector pcDNA 3 (In Vitrogen).

Synthetic Peptides.

Peptides were synthesized by a solid phase method using an AMS 422 multiple peptide synthesizer (Gilson Co., Worthington, OH) as described previously (29) . The amino acid sequences of the MART-1, gp100, and tyrosinase peptides used in this study are as follows: MART-1:27–35, AAGIGILTV (22) ; gp100:209–217, ITDQVPFSV (30) ; tyrosinase:1–9, MLLAVLYCL (31) ; tyrosinase:368–376 370 D, YMDGTMSQV (32) ; and tyrosinase:368–376 370 N, YMNGTMSQV (31) . The amino acid sequence of the hepatitis B virus core protein:18–27 23Y peptide (HBV:18–27 23Y) that was used for peptide binding assays was FLPSDYFPSV (33) . All peptides were dissolved in DMSO at 2 mg/ml.

Peptide Binding to HLA-A2.

The binding of peptides to HLA-A2 was measured using a live cell competitive binding assay as described, except T2 cells were substituted for EBV-transformed B cells (33) . Values obtained represented the IC_{50} for each peptide, which is the concentration of peptide that inhibits 50% of the binding of ^{125}I-labeled hepatitis B virus core protein residues 18–27 modified with a tyrosine at P6 (HBV:18–27 23Y) to HLA-A2 molecules on T2 cells. HBV:18–27 23Y was radiolabeled with ^{125}I using chloramine T, and labeled peptide was separated from the unincorporated ^{125}I using a Sephadex G-10 (Sigma) size exclusion column as described (33) . Binding of ^{125}I-labeled HBV:18–27 23Y peptide to T2 cells was measured in the presence of 10, 1, 0.1, 0.01, and 0.001 mg/ml unlabeled test peptide. The maximum binding and maximum inhibition values for each assay were determined by incubating T2 cells with ^{125}I-labeled HBV:18–27 23Y peptide alone or with an excess (50 mg/ml) of unlabeled HBV:18–27 23Y peptide. Each determination was performed in triplicate, and their average was used to calculate the percentage of inhibition as follows:

$$\%\ inhibition = 1 - \frac{Mean\ cpm_{experimental} - mean\ cpm_{maximum\ inhibition}}{Mean\ cpm_{maximum\ binding} - mean\ cpm_{maximum\ inhibition}} \times 100$$

The ln(% inhibition) was plotted *versus* the ln(peptide concentration). Using regression analysis, the IC_{50} was determined as the peptide concentration that inhibited 50% of the binding of the ^{125}I-labeled HBV:18–27 23Y peptide.

Synthetic Oligonucleotides.

Oligonucleotide primers were purchased from Life Technologies. The sequences of the human TCR BV subfamily-specific primers have been described elsewhere (34) . Human TCR BV subfamily-specific primers were designed based on the genomic TCR BV sequences reported by Rowen *et al.* (35) . Each primer was designed such that there was no more than a single base mismatch with any member of the subfamily. The official nomenclature proposed by the International Union of Immunological Societies subcommittee on nomenclature has been used to identify all TCR gene segments described (36) .

RNA Isolation and PCR.

Total cellular RNA was isolated from 5 x 10^6 TIL 1383 I cells using Trizol (Life Technologies, Inc., Gaithersburg, MD), and cDNA was synthesized using oligo $dT_{(12–18)}$ and Superscript II reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) as described (34) . Each TCR BV subfamily-specific forward primer was used in conjunction with a BC reverse primer to amplify the TCR BV

transcripts present in the TIL culture. PCR reactions consisted of 200 μM deoxynucleotide triphosphate (Oncor, Gaithersburg, MD), 400 nM of both forward and reverse primers, and 1 unit of Taq polymerase (Oncor, Gaithersburg, MD) in a 50-μl reaction. Amplifications were performed in a Perkin-Elmer 9600 DNA thermocycler (Norwalk, CT) under the following conditions: 35 cycles of 92°C denaturation for 30 s, 60°C annealing for 30 s, and 72°C extension for 1 min. PCR products were separated using ethidium bromide-stained agarose gels and electronically imaged using an Eagle Eye II Still Video System (Stratagene, La Jolla, CA).

DNA Sequence Analysis.

The DNA sequence analysis of the TCRBV12 band from TIL 1383 I was performed using the BV12a primer and Dye Terminator Cycle Sequencing kits (Perkin-Elmer/ABI, Foster City, CA). Sequences were determined using an ABI Prism 310 Genetic Analyzer (Perkin-Elmer/ABI). The resulting sequences were analyzed using the Genetics Computer Group, Inc. software package (37) .

Immunofluorescence and Cell Sorting.

Cell surface expression of TCRBV12, CD3, CD4, and CD8 molecules on TIL 1383 I was measured by immunofluorescence using FITC-conjugated anti-TCR BV12 (Immunotech, Westbrook, ME), PE-conjugated anti-CD3 (Becton Dickinson, San Jose, CA), FITC- or PE-conjugated anti-CD4 (Becton Dickinson), and FITC- or PE-conjugated anti-CD8 mAbs (Becton Dickinson). For analysis, the relative log fluorescence of 10^4 live cells was measured using a FACScan flow cytometer (Becton Dickinson). For cell sorting, the TCR BV12 mAb was dialyzed against PBS to remove the sodium azide and filter sterilized. Cells (2×10^7) cells from a 54-day-old TIL 1383 I culture were stained with FITC-conjugated anti-TCR BV12 mAb. BV12-expressing cells were collected using a Coulter Elite flow cytometer (Coulter, Miami, FL).

Tumor Antigen Recognition Assays.

The reactivity of TIL 1383 I was measured in cytokine release assays as described (38) . Briefly, TIL 1383 I cells were cocultured overnight with stimulator cells in a 1:1 ratio (the actual number of stimulators and responders used are indicated in the table legends) in RPMI 1640 supplemented with 10% heat-inactivated pooled human AB serum and 1% penicillin-streptomycin-glutamine in a total volume of 200 ml in 96-well U-bottomed plates. Supernatants were harvested, and the amount of cytokine release was measured by ELISA. IFN-γ, TNF-α, IL-4, and IL-10 ELISA kits were purchased from Endogen (Woburn, MA). GM-CSF and IL-2 ELISA kits were purchased from R&D Systems (Minneapolis, MN). The stimulators used in these assays included a panel of HLA-$A2^+$ and HLA-$A2^-$ tumors, EBV-transformed B cells, fibroblast lines, and peptide-pulsed T2 cells. Peptide-pulsed T2 cells were prepared by incubating 1×10^6 T2 cells/ml with peptide for 2 h at 37°C.

Transient Transfection Assays.

COS-7 cells were transiently transfected using Lipofectamine Plus (Life Technologies, Rockville, MD) as described (39) . Briefly, 5×10^4 COS-7 cells/well in 96-well, flat-bottomed plates were incubated in 40 ml of serum-free DMEM medium containing 200 ng of pcDNA 3 containing the cloned tumor-associated antigen cDNA (150 ng) and HLA-A2.1 (50 ng) cDNA at 37°C in a humidified CO_2 incubator (5% CO_2) for 3 h. Then, 160 ml of DMEM medium supplemented with 10% heat-inactivated fetal

bovine serum and 1% penicillin-streptomycin-glutamine was added. After 2 days, 100 ml of medium were removed, and 2.5 x 10^4 T cells in 100 ml of CM were added to each well. The amount of IFN-γ was measured by ELISA as described above.

Antibody Inhibition Assays.

The ability of anti-MHC, anti-CD4, and anti-CD8 mAbs to inhibit antigen recognition by TIL 1383 I was measured in cytokine release assays as described (38) . Briefly, 1 x 10^4 T cells and 1 x 10^4 tumor cells were cocultured in RPMI 1640 supplemented with 10% heat-inactivated pooled AB serum and 1% penicillin-streptomycin-glutamine in a total volume of 200 ml in 96-well U-bottomed plates for 24 h. For the MHC blocking assays, the tumor cells were incubated with IVA12 and/or W6/32 mAb (final concentration, 20 mg/ml) for 30 min at room temperature prior to the addition of the T cells. For the CD4/CD8 blocking assays, the T cells were incubated with anti-CD4 (final concentration, 2.5 mg/ml) or anti-CD8 (final concentration, 1.25 mg/ml) mAb for 30 min at room temperature prior to the addition of the tumor cells. The amount of IFN-γ released was measured by ELISA as described above.

^{51}Cr Release Assays.

The ability of TIL 1383 I to lyse HLA-A2$^+$, tyrosinase$^+$ target cells was measured in ^{51}Cr release assays as described (15) . Briefly, 10^6 melanoma or peptide-pulsed T2 cells were labeled for 1 h at 37°C with 200 mCi of ^{51}Cr (Amersham, Arlington Heights, IL) in CM. Labeled target cells (5 x 10^3) were incubated with 4 x 10^5 (80:1), 1 x 10^5 (20:1), 2.5 x 10^4 (5:1), and 6.25 x 10^3 (1.25:1) effector cells for 4 h at 37°C in 200 ml of CM. Supernatants were harvested and counted using an Wallac 1470 Wizard automatic gamma counter (Wallac, Gaithersburg, MD). Total and spontaneous ^{51}Cr release by each target was determined by incubating 5 x 10^3 labeled target cells in 2% SDS or CM, respectively, for 4 h at 37°C. Each point represented the average of triplicate wells, and percentage of specific lysis was calculated as follows:

$$\%\ specific\ lysis = \frac{Specific\ ^{51}Cr\ release - spontaneous\ ^{51}Cr\ release}{Total\ ^{51}Cr\ release - spontaneous\ ^{51}Cr\ release} \times 100$$

RESULTS

Twelve TIL cultures from patient 1383 were established from an enzymatically digested melanoma lesion by culturing the cells in medium containing 6000 IU/ml of recombinant human IL-2. Each culture was expanded separately and tested on day 17 for its reactivity against the autologous 1383 MEL, an HLA-A2$^+$ and an HLA-A2$^-$ melanoma cell line. One culture, designated TIL 1383 I, appeared to be HLA-A2 restricted and melanoma reactive in the initial screen (data not shown). TIL 1383 I was expanded, and its reactivity was determined using a more extensive panel of melanoma and nonmelanoma cell lines. As shown in Table 1⊡ , TIL 1383 I secreted significant amounts of IFN-γ (at least twice background and >100 pg/ml) when stimulated with 9 of the 11 HLA-A2$^+$ melanomas but with none of the five HLA-A2$^-$ melanomas (Table 1)⊡ . TIL 1383 I did not recognize any of the HLA-A2$^+$ or HLA-A2$^-$ EBV transformed B-cell,

fibroblast, prostate carcinoma, ovarian carcinoma, renal cell carcinoma, colon carcinoma, esophageal carcinoma, and breast carcinoma cell lines. These results indicated that TIL 1383 I recognized a shared melanoma antigen in the context of HLA-A2.

View this table: Table 1 HLA-A2-restricted recognition of melanoma cell lines by TIL 1383 I
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TIL 1383 I was then tested for its ability to recognize transfectants expressing a panel of melanoma antigens described previously containing known HLA-A2-restricted epitopes. As shown in Table 2, TIL 1383 I recognized COS cells that had been transiently transfected with the HLA-A2 and tyrosinase cDNAs. COS cells transfected with HLA-A2 and MART-1 were only recognized by TIL 1235, and COS cells transfected with HLA-A2 and gp100 were only recognized by TIL 1520. None of the TILs recognized COS cells transfected with HLA-A2 alone, HLA-A2 and TRP2, or HLA-A2 and Eso I. Recognition of the tumor cell controls in this assay was consistent with each of the TILs being HLA-A2 restricted and melanoma reactive. Therefore, the melanoma-associated antigen recognized by TIL 1383 I was tyrosinase.

View this table: Table 2 HLA-A2-restricted recognition of tyrosinase by TIL 1383 I
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Two HLA-A2-restricted tyrosinase epitopes consisting of amino acid positions 1–9 and 368–376 (designated T9-1 and T9-368, respectively) have been described previously (31) . To determine whether one of these two HLA-A2-restricted tyrosinase epitopes were recognized by TIL 1383 I, T2 cells pulsed with either T9-1 or T9-368 were assayed for their ability to stimulate IFN-γ release from TIL 1383 I. The T9-368 peptide undergoes a posttranslational modification that changes the N at position 370, which is encoded by the tyrosinase gene to a D, which is found on the surface of melanoma cells (32) . As shown in Table 3, TIL 1383 I recognized T2 cells pulsed with the T9-368 peptide with either N or D at position 370. TIL 1383 I recognized the HLA-$A2^+$ melanoma lines but not T9-1, M9-27, or G9-209 pulsed T2 cells, 1383 fibroblasts, or 888 MEL. TIL 1235 recognized M9-27, TIL 1520 recognized G9-209, and TIL 620 recognized both M9-27 and G9-209 as expected. TIL 888, which is known to recognize tyrosinase in the context of HLA-A24, recognized the 888 MEL lines but did not recognize any of the HLA-A2-restricted peptides pulsed on T2 cells. Therefore, the previously described tyrosinase epitope T9-368 was recognized by TIL 1383 I, with both the 370N and the 370D forms of the peptide being recognized efficiently.

View this table: Table 3 Recognition of the tyrosinase:368376 peptide by TIL 1383 I
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The TCR BV genes used by TIL 1383 I was determined by reverse transcription-PCR using TCR BV subfamily-specific primers as described (34) . TIL 1383 I was oligoclonal because we could only detect BV12, BV14, and BV24 transcripts, with BV12 being the predominant subfamily used (data not shown). DNA sequence analysis of the BV12 band found only a single productive rearrangement, indicating that the BV12 subfamily was clonally expanded in TIL 1383 I (data not shown). To confirm that $BV12^+$ T cells were the predominant T-cell clonotype in this culture, the expression of BV12, CD3, CD4, and CD8 on TIL 1383 I was measured by immunofluorescence. Essentially 100% of the T cells in TIL 1383 I expressed both CD3 and CD4 (Fig. 1*c*) , with ~99% of the $CD4^+$ T cells expressing BV12 (Fig. 1*d*) . Less than 1% of the TIL 1383 I cells were $CD8^+$ (Fig. 1*e*) or $CD8^+$ and $BV12^+$ (Fig. 1*f*) . These rare events were attributable to nonspecific staining by the anti-CD8 mAb because there were no $CD4^-$ T cells in TIL 1383 I (Fig. 1, *c* and *d*) , and we were unable to detect CD8a chain transcripts by reverse transcription-PCR (data not shown). We wanted to obtain a pure population of $BV12^+$, $CD4^+$ T cells from TIL 1383 I to determine whether these were the tyrosinase-reactive cells in the TIL 1383 I culture. Because attempts to clone TIL 1383 I in limiting dilution were unsuccessful, we stained a TIL 1383 I culture with an anti-BV12 mAb and used the cell sorter to further enrich the population of $BV12^+$ T cells. After two sorts, we were able to generate a TIL 1383 I culture that was 100% $BV12^+$ T cells (Fig. 2*c*) from a culture that was 98% $BV12^+$ T cells (Fig. 2*b*) . The sorted cells were expanded and tested for expression of BV12, CD4, and CD8 and for their ability to recognize antigen. After 2 weeks of culture, 100% of sorted cells remained $CD4^+$ and $BV12^+$ (data not shown). When tested for their antigen recognition, the sorted cells specifically recognized T9-368 pulsed T2 cells, and the amount of IFN-γ released was equivalent to the amount released by the unsorted cells (Table 4) . These results indicated that the HLA-A2-restricted recognition of tyrosinase by TIL 1383 I was by a BV12-expressing, $CD4^+$ T cell.

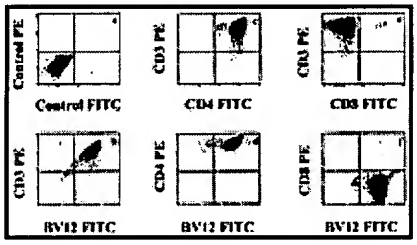

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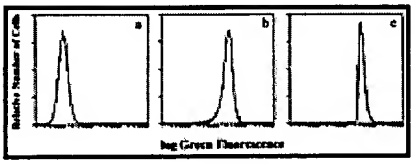
Fig. 1. Cell surface phenotype of TIL 1383 I. The percentage of TIL 1383 I cells expressing $CD3^+$, $CD4^+$, $CD8^+$, and $BV12^+$ was determined by immunofluorescence staining, followed by flow cytometry analysis. Each histogram represents the fluorescence profiles of at least 5×10^3 viable cells.

Fig. 2. Purification of $BV12^+$ T cells from TIL 1383 I. A day 52 TIL 1383 I culture was stained with anti-BV12-FITC mAb and sorted for high and uniform expression of $BV12^+$ T cells.



Each histogram contains the fluorescence profiles of 10^4 live unstained (*a*), BV12 stained but not sorted (*b*), or BV12 stained and twice sorted cells (*c*).

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View this table: Table 4 Recognition of the tyrosinase:368–376 peptide by BV12 sorted TIL 1383 I
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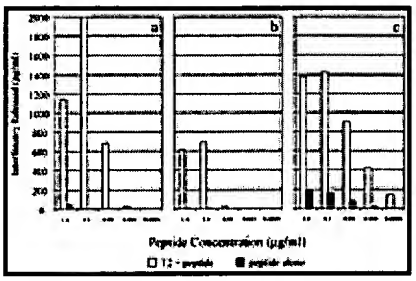
Human T cells differ from murine T cells in that activated human T cells express MHC class II molecules and can process and present class II-restricted epitopes to each other. Therefore, it is possible that the tyrosinase peptide could be shed from the tumor cells and presented by the TIL 1383 I cells to each other in the context of MHC class II rather than the MHC class I. To demonstrate that the T9-368 peptide was directly presented to TIL 1383 I cells by HLA-A2 on the tumor cells or T2 cells, we compared cytokine secretion by TIL 1383 I when stimulated with peptide-pulsed T2 cells to T cells stimulated with free peptide. TIL 1383 I secreted significant amounts of IFN-γ when stimulated with T2 cells pulsed with 1.0, 0.1, or 0.01 mg/ml of T9-368 370D peptide (Fig. 3*a*) and 1.0 or 0.1 mg/ml of T9-368 370N peptide (Fig. 3*b*). In contrast, TIL 1383 I did not secrete IFN-γ when stimulated with the T9-368 370D or 370N peptide alone. The $CD8^+$ T cell control TIL 1520 cells also secreted IFN-γ when stimulated with G9-209 pulsed T2 cells (Fig. 3*c*). TIL 1520 cells secreted a low but significant amount of IFN-γ when stimulated with free peptide (200 pg/ml with 1.0 mg/ml peptide). When the ability of these peptides to bind to HLA-A2 molecules was measured in a competition binding assay, the IC_{50} for G9-209, T9-368 370D, and T9-368 370N was measured to be 74, 702, and 249 mM, respectively. One possible explanation for the ability of free G9-209 peptide to stimulate TIL 1520 could be that the binding affinity of G9-209 was high enough to allow a small amount of peptide to be bound to the HLA-A2 molecules on the surface of the T cells. The TIL 1520 cells could then stimulate each other to secrete IFN-γ. In contrast, TIL 1383 I cells were unable to stimulate each other when cultured with free peptide, possibly because the low binding affinity of the T9-368 peptides did not allow efficient binding of peptide to the HLA-A2 molecules on their cell surface. These results indicate that the IFN-γ released by TIL 1383 I could only be attributable to the recognition of T9-368/HLA-A2 complexes on the tumor cells.

Fig. 3. IFN-γ secretion by TIL 1383 I requires T9-368 peptide presented by HLA-A2 molecules. TIL 1383 I cells (*a* and *b*) or TIL 1520 cells (*c*) were stimulated with various concentrations of peptide alone (■) or peptide-pulsed T2 cells (□. The peptide used to stimulate the TIL cells was T9-368



370D (*a*), T9-368 370N (*b*), and G9-209 (*c*).

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The MHC class I-restricted recognition of tyrosinase by TIL 1383 I was further demonstrated in mAb blocking assays. As shown in Table 5, anti-MHC class I mAb W6/32 inhibited TIL 1383 I recognition of 1383 MEL (35%; 184.8 to 120.4 pg/ml), 1558 MEL (46%; 220.7 to 119.1 pg/ml), and T9-368 pulsed T2 cells (61%; 1146.9 to 451.7 pg/ml). In contrast, anti-MHC class II mAb IVA12 had little effect on TIL 1383 I recognition of melanoma cells (1383 MEL: 9%, 184.8 to 167.3 pg/ml; 1558 MEL: 0%, 234.2 to 220.7 pg/ml) or peptide-pulsed T2 cells (17%; 1146.9 to 955.7 pg/ml). W6/32 inhibited MHC class I restricted IFN-γ release by control of the $CD8^+$ T cell lines TIL 1520 or TIL 1235, and IVA12 inhibited class II-restricted IFN-γ release by the control of $CD4^+$ T-cell line TIL 1558, as expected (Table 5). The ability of W6/32 to inhibit recognition of HLA-$A2^+$ $tyrosinase^+$ melanoma cells and peptide-pulsed targets by TIL 1383 I is a further demonstration of its MHC class I-restricted reactivity.

View this table: Table 5 Antibody inhibition of IFN-γ release by TIL 1383 I
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The contribution of the CD4 coreceptor to antigen recognition by TIL 1383 I was determined by blocking cytokine release with anti-CD4 mAb. The amount of IFN-γ released by TIL 1383 I when stimulated with 1558 MEL was reduced by 15% (705.4 to 604.9 pg/ml) and 23% (705.4 to 546.5 pg/ml) in the presence of anti-CD4 mAb and anti-CD8 mAb, respectively (Table 5). Anti-CD4 mAb inhibited MHC class II-restricted recognition of 1558 MEL by $CD4^+$ TIL 1558 (44% inhibition; 1651.0 to 930.0 pg/ml) but had no effect on MHC class I-restricted recognition of 1558 MEL by TIL 1520 (4% inhibition; 507.2 to 489.5 pg/ml). Anti-CD8 mAb inhibited MHC class I-restricted recognition of 1558 MEL by $CD8^+$ TIL 1520 (46% inhibition; 507.2 to 276.0 pg/ml) but had minimal effects on MHC class II-restricted recognition of 1558 MEL by TIL 1558 (17% inhibition; 1651.0 to 1363.0 pg/ml). These results indicate that the CD4 coreceptor is not required for antigen recognition by TIL 1383 I.

The pattern of cytokine release from TIL 1383 I after antigen stimulation is shown in Table 6. TIL 1383 I secreted IFN-γ, GM-CSF, TNF-α, and IL-2 but not IL-4 or IL-10 when stimulated with either the autologous 1383 MEL, 1558 MEL, or OKT3. By comparison, $CD8^+$ TIL 1520 secreted only IFN-γ and GM-CSF when stimulated with 1383 MEL or 1558 MEL and IFN-γ, GM-CSF, and TNF-α when

stimulated with OKT3. In this experiment, 1383 fibroblast-stimulated TIL 1383 I secreted <20% of the amount of GM-CSF secreted by TIL 1383 I stimulated with tumor cells. Fibroblasts alone secreted small amounts of TNF-α and GM-CSF. These results indicate that the pattern of cytokine secretion by TIL 1383 I was consistent with a Th_1 phenotype, despite being an MHC class I-restricted $CD4^+$ T cell.

View this table: Table 6 Cytokine release profile of TIL 1383 I
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The ability of TIL 1383 I to lyse HLA-$A2^+$ melanoma cells and T9-368 pulsed T2 cells was tested in 4-h ^{51}Cr release assays. As shown in Fig. 4*a*, TIL 1383 I weakly lysed 888 A2 MEL and T9–368 370D pulsed T2 cells but failed to lyse autologous 1383 MEL, 1383 fibroblasts, and M9–27 pulsed T2 cells. TIL 888, which recognizes tyrosinase in the context of HLA-A24, efficiently lysed only the HLA-$A24^+$ melanoma lines 888 MEL and 888 A2 MEL (Fig. 4*b*). TIL 1235 efficiently lysed 888 A2 MEL and M9-27-pulsed T2 cells but not 1383 MEL, 1383 fibroblasts, and T9-368 pulsed T2 cells (Fig. 4*c*). The failure of 1383 MEL to be lysed by TIL 1235 suggested that 1383 MEL may be resistant to cell-mediated lysis. Therefore, despite being a $CD4^+$ T cell, TIL 1383 I could weakly lyse some of the HLA-$A2^+$, tyrosinase$^+$ targets.

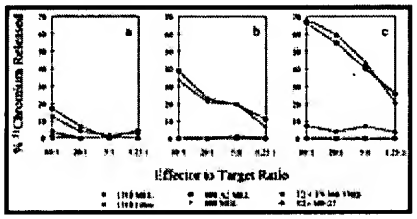

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Fig. 4. Lysis of HLA-$A2^+$ melanoma cells by TIL 1383 I. Lysis of melanoma and peptide-pulsed T2 cells by TIL 1383 I (*a*), TIL 888 (*b*), and TIL 1235 (*c*) was measured in standard 4-h ^{51}Cr release assays. Targets included 1383 MEL (•), 1383 fibroblasts (x), 888 MEL (+), 888 A2 MEL (■), T9-368 370D (♦), and M9-27 (▲) pulsed T2 cells.

DISCUSSION

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Classically, $CD4^+$ T cells recognize antigens presented by MHC class II molecules, whereas $CD8^+$ T cells recognize antigens presented by MHC class I molecules (3, 4, 5). Recently, several examples of MHC class I-restricted $CD4^+$ T cells have been reported (40, 41, 42, 43, 44, 45, 46). However, the existence of MHC class I-restricted antigen recognition by $CD4^+$ T cells has remained controversial because no single study has been able to exclude the presence of contaminating $CD8^+$ T cells in the culture, determine the MHC class I restriction element, and identify the nominal antigen recognized by the T cells. In this study, we

described an MHC class I-restricted $CD4^+$ T cell isolated from the infiltrating lymphocytes of a human melanoma lesion. Immunofluorescence analysis indicated that 100% of the cells in the bulk TIL 1383 I culture and cells sorted for high expression of BV12 were $CD3^+CD4^+$ T cells with no evidence of contaminating $CD8^+$ T cells (Figs. 1 and 2). The reactivity of TIL 1383 I with a broad panel of tumor targets indicated that the antigen recognized by TIL 1383 I was melanoma associated and HLA-A2 restricted (Table 1). The antigen recognized by TIL 1383 I was tyrosinase, with the 368–376 peptide being the antigenic peptide (Tables 2 and 3). Identification of the antigenic peptide enabled us to conclusively demonstrate that a ligand for the TCR expressed by the $CD3^+CD4^+BV12^+$ T cell in TIL 1383 I was the T9-368 peptide derived from tyrosinase bound to HLA-A2 (Fig. 3).

Current models of MHC restriction, antigen specificity, and T-cell development require that T cells are positively selected by peptide/MHC molecules expressed on the host thymic epithelial cells (47, 48). From these models, there are several potential ways an MHC class I-restricted $CD4^+$ could have developed. One possibility is that TIL 1383 I was positively selected by T9–368/HLA-A2 complexes expressed on the thymic epithelium. Peptides presented by MHC class I molecules are generally derived from proteins encoded endogenously by the antigen-presenting cell, implying that all of the peptides involved in the positive selection of $CD8^+$ T cells must be derived from proteins encoded by the thymus. The expression of tyrosinase is limited to cells of the melanocyte/melanoma lineage (49). Unless the T9-368 peptide was shed by melanocytes and exchanged with peptides on MHC class I molecules in the thymus, T9-368/HLA-A2 complexes should not exist in the thymus and would therefore be unavailable to positively select TIL 1383 I. However, we cannot exclude the formal possibility that TIL 1383 I was positively selected by some cross-reactive, thymus-derived peptide presented by HLA-A2. It is also possible that TIL 1383 I could be functionally a $CD4^+$ $CD8^+$ T cell that carries a mutant CD8 chain that fails to bind our anti-CD8 mAb, making TIL 1383 I appear to be a $CD4^+CD8^-$ T cell. However, the model that is most consistent with the current theories of positive selection is that TIL 1383 I was positively selected by some undefined peptide in association with an MHC class II molecule in this patient, and the HLA-A2-restricted recognition of the T9-368 peptide is merely an unusual cross-reaction.

Cross-reactivity is commonly observed among mAbs but has also been attributed to T cells (6, 7, 12, 13, 14). In the immune response to human melanoma, epitope mimicry was directly investigated by determining the ability of MART-1-reactive CTLs to recognize a panel of naturally occurring peptide mimics derived from human self antigens and pathogens (12). Many of these peptides were recognized by the MART-1-reactive CTL clones, and their pattern of peptide recognition (which peptides were recognized and the magnitude of the response) was unique to each T-cell clone. It is, therefore, possible that epitope mimics of the T9-368 peptide exist that can be recognized by TIL 1383 I or that the T9-368 peptide is itself a mimic of some other unknown peptide.

We believe that the variation in the way each T-cell clone recognizes antigen is reflected by the variation in the TCR V genes used by the T-cell clone. Epitope mimicry should then lead to TCR diversity in the immune response to a single antigenic peptide. Despite reports of restricted TCR V gene usage by melanoma reactive T cells (50), we have found TCR β chain usage by melanoma-reactive T cells to be

quite diverse (34 , 51 , 52) . In one patient, as many as 10 distinct TCR clonotypes using seven different TCR BV genes were used to recognize the same gp100:209–217/HLA-A2 complex.[3] These observations are consistent with the hypothesis that the vast TCR diversity we observe among melanoma-reactive T cells may be attributable to the broad cross-reactivity and redundancy of the immune system.

If epitope mimicry can lead to $CD4^+$ T cells that recognize antigen in the context of MHC class I molecules, then T cells should be more common than they appear. There are probably two main reasons why there are very few reported examples of class I-restricted $CD4^+$ T cells: (*a*) most class I-restricted T cells are isolated based on their ability to lyse ^{51}Cr-labeled targets. Because most $CD4^+$ T cells are not cytolytic, class I-restricted $CD4^+$ T cells would be generally overlooked in lysis assays. In fact, had we used lysis assays as our initial screen, TIL 1383 I would have been classified as nonreactive and discarded because it failed to lyse the autologous melanoma (Fig. 4); and (*b*) most T cells require the CD4 or CD8 coreceptors to respond to antigen stimulation (53 , 54) . CD4 binds to the b2 domain of MHC class II molecules (55) , and CD8 binds to the a3 domain of MHC class I molecules (56) . The function of these coreceptors is to stabilize the TCR/peptide/MHC complex (57 , 58) and enhance T-cell signaling by augmenting CD3z and ZAP70 phosphorylation (59) . However, there is T-cell to T-cell variation in the requirement for CD8 for T-cell function (60) . Those T cells whose function is CD8 dependent have low- to intermediate-affinity TCRs, and those T cells whose function is CD8 independent have high-affinity TCRs (61 , 62) . CD4 and CD8 independent clones are relatively rare, suggesting that T cells bearing high-affinity TCRs are relatively rare. Our inability to block T9-368 peptide or tumor cell recognition with anti-CD4 mAb, together with incomplete blocking of IFN-γ secretion by anti-MHC class I mAb, strongly suggests that the BV12 TCR expressed by TIL 1383 I must have sufficient affinity for the T9-368/HLA-A2 complex that it can activate the T cell in the absence of the CD4 coreceptor. Despite having a high-affinity TCR, TIL 1383 I has relatively low avidity compared with other class I-restricted T cells (recognized 10 nM peptide *versus* ≤ 1 nM peptide; Fig. 3).

TIL 1383 I may prove to be a useful reagent for addressing basic questions of T-cell development and immunological tolerance. We and others have shown that the transfer of low-affinity, MHC class I-restricted TCRs with apparent low affinity into cells that lack the CD8 coreceptor result in cells with low avidity (63, 64, 65) . Coexpression of CD8 increased the avidity of the T cells, resulting in enhanced antigen recognition (64 , 65) . It is interesting to note that the reactivity of TIL 1383 I is similar to the reactivity of T9-368-reactive $CD8^+$ T cells. In both cases, the T cells had higher avidity for the T9-368 370D peptide, which is expressed on the surface of melanoma cells than the T9-368 370N peptide, which is encoded by the tyrosinase gene (32) . Therefore, the only apparent difference between T9-368-reactive $CD8^+$ T cells and TIL 1383 I is the high-affinity TCR expressed by TIL 1383 I. Because high avidity T cells appear to be more effective in treating virus-infected mice than low avidity T cells (66) , expressing this high-affinity TCR in normal $CD8^+$ T cells should result in high avidity T cells that may be more effective in eliminating metastatic disease. Another possible benefit of this high-affinity TCR would be to transfer this MHC class I reactivity to normal $CD4^+$ T cells. Given that TIL 1383 I recognized tumor cells without any contribution from the CD4 coreceptor, we would predict peripheral blood lymphocyte-derived $CD4^+$ T cells bearing this TCR would also recognize $HLA\text{-}A2^+$, $tyrosinase^+$

melanoma cells. The ability of tumor-reactive $CD4^+$ T cells to mediate tumor regression could be evaluated in a greater number of patients because HLA-A2 is expressed by ~50% of all melanoma patients.

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FOOTNOTES

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[1] To whom requests for reprints should be addressed, at Surgery Branch, National Cancer Institute, NIH, Building 10/2B06, 9000 Rockville Pike, Bethesda, MD 20892. Phone: (301) 402-4123; Fax: (301) 402-1788; E-mail: nishimur@helix.nih.gov.

[2] The abbreviations used are: TIL, tumor infiltrating lymphocyte; IL, interleukin; GM-CSF, granulocyte/macrophage-colony stimulating factor; TNF, tumor necrosis factor; CM, complete medium; TCR, T-cell receptor; PE, phycoerythrin; mAb, monoclonal antibody.

[3] Unpublished observations.

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